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The *Pseudomonas syringae* pv. tomato HrpW Protein Has Domains Similar to Harpins and Pectate Lyases and Can Elicit the Plant Hypersensitive Response and Bind to Pectate

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The host-specific plant pathogen *Pseudomonas syringae* elicits the hypersensitive response (HR) in nonhost plants and secretes the HrpZ harpin in culture via the Hrp (type III) secretion system. Previous genetic evidence suggested the existence of another harpin gene in the *P. syringae* genome. *hrpW* was found in a region adjacent to the *hrp* cluster in *P. syringae* pv. tomato DC3000. *hrpW* encodes a 42.9-kDa protein with domains resembling harpins and pectate lyases (Pels), respectively. HrpW has key properties of harpins. It is heat stable and glycine rich, lacks cysteine, is secreted by the Hrp system, and is able to elicit the HR when infiltrated into tobacco leaf tissue. The harpin domain (amino acids 1 to 186) has six glycine-rich repeats of a repeated sequence found in HrpZ, and a purified HrpW harpin domain fragment possessed HR elicitor activity. In contrast, the HrpW Pel domain (amino acids 187 to 425) is similar to Pels from *Nectria haematococca*, *Erwinia carotovora*, *Erwinia chrysanthemi*, and *Bacillus subtilis*, and a purified Pel domain fragment did not elicit the HR. Neither this fragment nor the full-length HrpW showed Pel activity in A_{230} assays under a variety of reaction conditions, but the Pel fragment bound to calcium pectate, a major constituent of the plant cell wall. The DNA sequence of the *P. syringae* pv. syringae B728a *hrpW* was also determined. The Pel domains of the two predicted HrpW proteins were 85% identical, whereas the harpin domains were only 53% identical. Sequences hybridizing at high stringency with the *P. syringae* pv. tomato *hrpW* were found in other *P. syringae* pathovars, *Pseudomonas viridiflava*, *Ralstonia* (*Pseudomonas*) *solanacearum*, and *Xanthomonas campestris*. Δ*hrpZ*::*nptII* or *hrpW*::ΩSp^r^ *P. syringae* pv. tomato mutants were little reduced in HR elicitation activity in tobacco, whereas this activity was significantly reduced in a *hrpZ hrpW* double mutant. These features of *hrpW* and its product suggest that *P. syringae* produces multiple harpins and that the target of these proteins is in the plant cell wall.

Pseudomonas syringae is a plant pathogen whose individual strains are classified into pathovars largely on the basis of host specificity. In incompatible or nonhost plants, *P. syringae* elicits the plant defense-associated hypersensitive response (HR), a rapid, localized, active death of plant cells that are in contact with bacteria (15, 33). As is characteristic of the common gram-negative plant-pathogenic bacteria, elicitation of the HR in nonhosts or pathogenesis in hosts is dependent on *hrp* genes (3, 36). Nine of these have recently been renamed *hrc* to indicate that they encode conserved components of a type III (host contact-dependent) secretion pathway that animal pathogens such as *Yersinia* spp. and plant pathogens such as *Pseudomonas* and *Xanthomonas* spp. apparently use to introduce pathogen proteins into host cells (3, 9, 14). Genes encoding the type III pathway are clustered on plasmids or in pathogenicity islands containing related virulence functions (21). Cosmid pHIR11, cloned from *P. syringae* pv. syringae 61, carries all the genes necessary for nonpathogenic bacteria such as *Pseudomonas fluorescens* and *Escherichia coli* to elicit the HR in tobacco (but not to cause disease) (26). These include genes encoding positive regulatory factors, the type III secretion pathway, and HrpZ and HrmA, two proteins thought to travel the pathway (4, 19, 23, 26, 48).

Three classes of proteins that are secreted by plant-pathogenic bacteria and have strong effects on plants have been extensively studied. (i) Pectic enzymes, especially pectate lyase (Pel) isozymes, cleave α-1,4-galacturonosyl linkages in plant cell wall pectic polymers, resulting in tissue maceration and death of the constituent cells due to osmotic fragility (12). Host-promiscuous, macerating pathogens such as *Erwinia chrysanthemi* and *Erwinia carotovora* secrete copious amounts of several Pel isozymes by the type II (Sec-dependent) pathway (7). However, Pel production by *P. syringae* seems to have little role in pathogenesis (8). (ii) Harpins, such as the *Erwinia amylovora* HrpN and *P. syringae* HrpZ proteins, are glycine-rich, cysteine-lacking proteins that are secreted in culture when the Hrp (type III) system is expressed and possess heat-stable HR elicitor activity when infiltrated into the leaf intercellular spaces of tobacco and several other plants (2, 23, 47). (iii) Avr proteins are so named because their presence in an Hrp^+^ bacterium triggers the HR defense in plants carrying a corresponding *R* gene, thus rendering the pathogen avirulent. Avr proteins are not secreted in culture and have no apparent effect when infiltrated into the intercellular spaces of leaves. There is now strong but indirect evidence that many Avr proteins are transferred to the interior of plant cells by the Hrp systems of *Pseudomonas* and *Xanthomonas* spp. and that at least one pair of *avr-R* gene products (AvrPto-Pto) physically interact within the plant cell cytoplasm (19, 35, 43, 45, 46). According to a current model for *P. syringae*-plant interactions,

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multiple Avr proteins are transferred into plant cells where they either collectively promote parasitism or individually betray the parasite to the host *R* gene surveillance system (2).

The activity of the *P. syringae* HrpZ harpin in HR elicitation is puzzling in many ways. A nonpolar *hrpZ* mutation causes a strong reduction in the HR phenotype of *E. coli*(pHIR11) but only a weak reduction in the HR phenotype of *P. syringae* (1). This suggests that *P. syringae* pv. syringae carries at least one other gene outside of the region cloned in pHIR11 whose product functions similarly to HrpZ. Furthermore, it appears that the Avr-like HrmA, and not HrpZ, is responsible for the HR elicited by nonpathogenic bacteria carrying pHIR11 (1, 4). Finally, nonoverlapping fragments of HrpZ possess elicitor activity, and expression of the gene in *trans* in wild-type bacteria reduces rather than enhances HR elicitation (1).

P. syringae pv. tomato DC3000 offers several experimental advantages over *P. syringae* pv. syringae 61 for searching for a second harpin. DC3000 is a pathogen of both tomato and the model plant *Arabidopsis thaliana*; its *hrpZ* locus also has been cloned and characterized; the bacterium has been shown to secrete, in an Hrp-dependent manner, four proteins in addition to HrpZ; and the region flanking the *hrp* cluster, which contains the *avrE* locus, has been partially characterized (10, 38, 41, 49). We report here the cloning, mutagenesis, and analysis of the *P. syringae* pv. tomato DC3000 *hrpW* gene, which encodes a protein that is secreted by the Hrp pathway, is capable of eliciting the HR, and, surprisingly, has features of both harpins and Pels.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* strains were routinely grown in LM (22) or Terrific broth (42) at 37°C. The *E. coli* strains primarily used for plasmid constructions were DH5α and DH5αF'IQ (Life Technologies, Grand Island, N.Y.). For standard DNA manipulations, the pBluescript II vectors from Stratagene (La Jolla, Calif.) were used. *P. syringae* pv. tomato DC3000 (41) and *P. fluorescens* 55 (26) were grown in King's B broth (32) or in *hrp*-derepressing fructose minimal medium (27) at 30°C. Antibiotics were used at the following concentrations (μg/ml): ampicillin, 100; gentamicin, 10; kanamycin, 50; rifampin, 100; spectinomycin, 50; and tetracycline, 20.

DNA manipulations. DNA manipulations and PCR were performed according to standard protocols (28, 42). Oligonucleotide primers for sequencing or PCR were purchased from Life Technologies. PCR was performed with *Pfu* polymerase (Stratagene). All DNA sequencing was done at the Cornell Biotechnology Center with an Automated DNA Sequencer, model 373A (Applied Biosystems, Foster City, Calif.). DNA sequence was analyzed with Genetics Computer Group version 7.3 (17) and DNASTAR (Madison, Wis.) software packages.

Plant assays. Tobacco (*Nicotiana tabacum* L. 'Xanthi') and tomato (*Lycopersicum esculentum* Mill. 'Moneymaker') plants were grown and inoculated with bacteria as described previously (19). For virulence assays, bacterial suspensions containing 10^4 cells/ml were infiltrated into tomato leaves and monitored daily over a 5-day period for symptom development and bacterial multiplication.

Isolation of DNA flanking the *hrp* cluster in *P. syringae* pv. tomato DC3000 and B728a. A library of total DNA from *P. syringae* pv. tomato DC3000, partially digested with *Sau*3A, was constructed in cosmid vector pCPP47 (8). Hybridization at high stringency with ^{32}P-labeled *Pst*I fragments containing *hrpK* and *hrpR* from *P. syringae* pv. syringae 61 yielded several cosmids. A 6.5-kb *Eco*RI fragment from pCPP2357 (hybridizing with *hrpR*) was subcloned into pML123 (34), producing pCPP2373. pCPP2374 and pCPP2375 were constructed by partially digesting pCPP2373 with *Mfe*I and inserting an *Eco*RI fragment carrying the ΩSp^r fragment from pHP45Ω into transcription units IV and V, respectively (40). A *P. syringae* pv. syringae B728a cosmid library was provided by D. W. Bauer, and approximately 5,000 colonies were probed with a ^{32}P-labeled *P. syringae* pv. syringae 61 *hrcC* fragment. Restriction mapping and DNA sequencing were used to identify cosmid pCPP2346, which contains *hrpW*.

DNA gel blots. Total DNA (2 μg) was digested with *Eco*RI and separated by electrophoresis on 0.5% agarose gels. DNA was transferred to Immobilon-N Membrane (Millipore Co., Bedford, Mass.) and hybridized at 62°C for 8 h in HYB-9 DNA Hybridization Solution (GENTRA Systems, Research Triangle Park, N.C.) with a 1.3-kb PCR-amplified *hrpW* fragment that was labeled with ^{32}P by using the Prime-It II kit (Stratagene). The membranes were washed four times in 1.0% sodium dodecyl sulfate (SDS) and 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) followed by two washes in 1.0% SDS and 0.2× SSC. Membranes were exposed to OMAT X-ray film (Eastman Kodak, Rochester, N.Y.) for 4 to 12 h.

HrpZ and HrpW secretion assays. *P. fluorescens* 55 strains carrying pHIR11 and pML123 constructs containing the *hrpW* region were grown overnight in 2 ml of King's B plus appropriate antibiotics. The cultures were washed once with *hrp* gene-derepressing fructose minimal medium and resuspended in 15 ml of the same medium to an optical density at 600 nm of 0.4. Cultures were grown with moderate shaking at room temperature. After 12 h, the cells were collected by centrifugation in a Sorvall SS-34 rotor at 3,000 × *g* and resuspended up to 500 μl with water plus 20 μl of 100 mM phenylmethylsulfonyl fluoride (PMSF). Twenty microliters of 100 mM PMSF was added to the culture supernatants, and the supernatants were centrifuged at 25,000 × *g* for 30 min to pellet the remaining cells. The supernatant proteins were concentrated 100-fold with Centricon-10 concentrators (Amicon, Inc., Beverly, Mass.). SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot analyses, using previously obtained anti-HrpZ antibodies (23, 49) and the Western light chemiluminescent detection system (Tropix, Bedford, Mass.) and OMAT X-ray film (Eastman Kodak), were performed as previously described (1).

Preparation of HrpW and derivatives. A *Mfe*I-*Xho*I fragment from pCPP2357 containing *hrpW* was ligated to the *Eco*RI-*Xho*I sites of pET21(+) (Novagen, Madison, Wis.) to make pCPP2417. The complete coding sequence for HrpW was PCR amplified from pCPP2417 with the primers 5'-ATGAGGATCCAGC ATCGGCATCACACCC-3' (named W1) and 5'-ATGAAAGCTTAAGCTCG GTGTGTTGGGT-3' (named W2), which contained *Bam*HI and *Hin*dIII sites, respectively. DNA encoding the N-terminal 186 amino acids of HrpW was PCR amplified from pCPP2417 with the W1 primer and the primer 5'-ATGAAAGC TTGCCACCGCCTGTTGCAGT-3', which contained a *Hin*dIII site. DNA encoding the C-terminal 236 amino acids of HrpW was PCR amplified from pCPP2368 with the primer 5'-ATGAGGATCCGAGGGTGGCGTAACACCG-3', which contained a *Bam*HI site and the W2 primer. Amplified products corresponding to full-length HrpW and the N-terminal and C-terminal portions of HrpW were directionally cloned into the *Bam*HI and *Hin*dIII sites of pQE30 (Qiagen) resulting in pCPP2377, pCPP2378, and pCPP2379, respectively. Procedures used to isolate His-tagged proteins with Ni-nitrilotriacetic acid (NTA) spin columns (Qiagen) were as described previously (1). *E. coli* ABLE K (Stratagene) grown on M9 medium (42) and supplemented with glucose (0.2%), casamino acids (0.02%), and thiamine (1 μg/ml) was used to obtain His_6-HrpW because of apparent toxicity. SDS-PAGE and immunoblot analyses, using previously obtained anti-HrpZ and anti-HrpW antibodies (23, 49) and the Western light chemiluminescent detection system (Tropix) and OMAT X-ray film (Eastman Kodak), were performed as described previously (1).

Preparation of calcium pectate beads and pectate binding assays. One hundred millimolar $CaCl_2$ was added dropwise to vigorously stirring 0.2% (wt/vol) sodium pectate (Sigma, St. Louis, Mo.) dissolved in 100 mM Tris (pH 8.0) or 50 mM MES (morpholineethanesulfonic acid) (pH 5.6) to make calcium pectate beads. The beads were pelleted by low-speed centrifugation and resuspended in 2 volumes of buffer. For binding assays, 50 to 500 μg of protein was mixed with 500 μl of pectate bead mix and incubated for 30 min to 12 h at room temperature. The beads were pelleted by centrifugation, washed several times in 500 μl of buffer, and resuspended to 500 μl in buffer. Aliquots of 20 μl were taken from the bead mix prior to centrifugation, from the wash buffer at each step, and from the washed beads and then analyzed with SDS-PAGE to determine protein distribution. Calcium alginate beads were similarly prepared from medium-viscosity sodium alginate (Sigma), and binding assays were performed as described above for pectate. Tests for the ability of either calcium chloride or pectate to precipitate HrpW were conducted with and without the addition of 0.2% (wt/vol) hydrated beads of agarose (Agarose IV; Amresco, Solon, Ohio).

Construction of *hrpZ* and *hrpW* marker exchange mutations in *P. syringae* pv. tomato DC3000. To construct the *hrpZ* mutation, a 603-bp *Cla*I fragment internal to *hrpZ* was deleted from pCPP2334, a LITMUS 28 (New England Biolabs) derivative that contains *hrpA* and *hrpZ*, producing pCPP2336. An *nptII* derivative lacking a transcriptional terminator was PCR amplified from pCPP2988 (1) with the primers 5'-CCATCGATGGTGGTGGCGATAGCTAGACTGG-3' and 5'-CCATCGATGGTCTCGTGATGGCAGGTTG-3' and cloned into the unique *Cla*I site of pCPP2336 in the correct orientation. A *Bgl*II-*Hin*dIII fragment from the resulting construct, pCPP2338, carrying the *hrpZ* mutation was exchanged for the *Bgl*II-*Hin*dIII fragment, carried in pCPP2340, producing pCPP2342. A 5.3-kb *Eco*RI fragment from pCPP2342 that carried the *hrpZ* mutation was cloned into the broad-host-range plasmid, pRK415 (30), producing pCPP2344. An 8.5-kb *Eco*RI fragment from pCPP2375, which carried *hrpW* interrupted with an ΩSp^r fragment, was subcloned into pRK415, producing pCPP2376. Separately, pCPP2376 and pCPP2344 were electroporated into *P. syringae* pv. tomato DC3000. Loss of the plasmid and retention of the marker were achieved as previously described (1).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited in GenBank under accession numbers AF005221 (*P. syringae* pv. tomato *hrpW*) and AF037983 (*P. syringae* pv. syringae *hrpW*).

RESULTS

***hrpW* expressed in *trans* eliminates the ability of *P. fluorescens*(pHIR11) to elicit the HR.** To identify any harpin-like

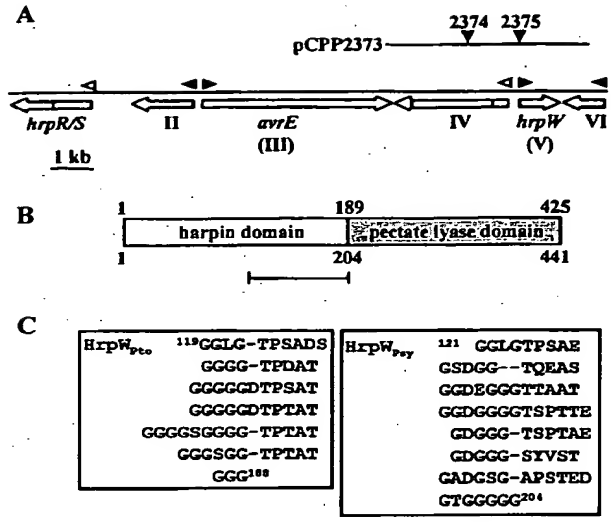

FIG. 1. Physical map of the *P. syringae* pv. tomato DC3000 *hrpW* region and structural features of HrpW. (A) The physical map of the DC3000 genome adjacent to the *hrp* gene cluster is shown with open arrowheads denoting putative σ^{54} promoters and with filled arrowheads denoting putative HrpL-dependent promoters that control previously defined transcriptional units (38). ORFs in polycistronic operons are marked by divisions in the open arrows denoting the operons. *hrpR* and *hrpS* encode regulatory proteins and are located at the right end of the *hrp* cluster. The region carried in pCPP2373 is indicated above the physical map, and inverted filled triangles mark the location of the ΩSp^r insertions in pCPP2374 and pCPP2375. (B) The diagram of HrpW indicates the harpin-like and Pel-like domains, with numbers on the top corresponding to DC3000 and on the bottom to B728a. The DC3000 *hrpW* PCR subclone-generated His_6-tagged harpin domain fragment encompasses amino acids 1 to 186; the His_6-tagged Pel domain has amino acids 187 to 425. (C) The sequences of the region in the middle of the HrpW proteins from *P. syringae* pv. tomato DC3000 (HrpW$_{Pto}$) and *P. syringae* pv. syringae B728a (HrpW$_{Psy}$), which contains glycine-rich repeats (denoted by the bar in panel B), are shown in the boxes. Dashes were inserted where necessary to permit alignment.

genes in the *P. syringae* pv. tomato DC3000 DNA flanking the *hrp-hrc* gene cluster, we isolated cosmid pCPP47 derivatives containing inserts hybridizing with *hrpK* or *hrpR*. These two genes define the left and right borders of the cluster. Subclones were constructed in pML123 and screened for two potential harpin phenotypes: (i) the ability to promote tobacco HR elicitation activity in *P. fluorescens* cells carrying pCPP2274, a Δ*hrpZ* pHIR11 derivative (19), and (ii) interference with the HR elicitation activity of *P. fluorescens* cells carrying wild-type pHIR11 (1). No subclones had the first phenotype, but subclone pCPP2373 had the second (data not shown). pCPP2373 contains a 6.5-kb *Eco*RI fragment from cosmid pCPP2357 (Fig. 1A), which hybridized with *hrpR* and has transcriptional units IV and V, which were previously identified and partially sequenced by Lorang and Keen (38). To determine which transcriptional unit in pCPP2373 was responsible for eliminating the HR elicitation activity of *P. fluorescens*(pHIR11), an ΩSp^r fragment was inserted into *Mfe*I sites in transcriptional units IV and V to construct pCPP2374 and pCPP2375, respectively (Fig. 1A). Both plasmids were transformed into *P. fluorescens*(pHIR11) cells, which were then infiltrated into tobacco leaves. Only pCPP2374 blocked HR elicitation, indicating that transcriptional unit V encoded a protein with one of the characteristics of HrpZ. HrpZ was produced in all strains but was not secreted in strains expressing transcriptional unit V, indicating that the block in HR elicitation was not due to negative regulation of *hrpZ* by transcriptional unit V (data not shown).

The DNA sequence of *hrpW* predicts a protein with both harpin and Pel domains. The complete DNA sequence of transcriptional unit V was determined for *P. syringae* pv. tomato DC3000, revealing a 1,275-bp open reading frame (ORF) that was designated *hrpW*. The gene is preceded by a consensus *hrp* promoter (38) and followed by a rho-independent terminator. The predicted N-terminal sequence of HrpW matches that of EXP-60, one of the five *P. syringae* pv. tomato DC3000 Hrp-secreted proteins identified by Yuan and He (49). *hrpW* is flanked by operons transcribed in divergent directions and appears to be in a monocistronic operon. Like harpins, the predicted 42.9-kDa HrpW protein is acidic and glycine rich, lacks cysteine, and is deficient in aromatic amino acids. The predicted protein sequence of HrpW reveals at least two distinct domains (Fig. 1B). A harpin-like domain (amino acids 1 to 189) is rich in glutamine, serine, and glycine. The region from amino acids 119 to 186 contains six imperfect glycine-rich repeats with many acidic and polar residues (Fig. 1C). The TPS/DAT motif in this region is predicted to have β-sheet structure with one side of the β-sheet having all the threonine and serine residues, and the glycine repeats are predicted to be turns by the Garnier-Robson algorithm (39). The alternating β-sheets and turns could form a β-barrel structure. Database searches done with BLAST (5) revealed no proteins with significant similarity to the harpin domain.

In contrast, the 236 C-terminal amino acids of HrpW are similar to several fungal Pels from *Nectria haematococca* mating type IV (*Fusarium solani* f. sp. *pisi*), to two bacterial Pels from *E. carotovora* and *E. chrysanthemi* (20, 37, 44), and to one bacterial Pel homolog from *Bacillus subtilis*. For example, HrpW shows identities of 32.5% with *N. haematococca* PelC (20), and 21.2% with Pel-3 from *E. carotovora* (37). HrpW retains two characteristics of harpins not found in homologous Pels: (i) all eight conserved cysteines of the Pels have been changed to other amino acids in the HrpW Pel-like region; and (ii) the predicted pI of the HrpW Pel region, 4.86, is much lower than the predicted pIs of its homologs, which range from 7.15 to 9.09. The *B. subtilis* Pel homolog, YvpA, which has not been shown to have Pel activity, has characteristics of both the HrpW Pel domain and active Pels; it is missing all but one cysteine, but has a basic pI. The amino acid sequence of Pels in this group is dissimilar to those of the majority of known Pels, and little is known about the active site or tertiary structure of this group of proteins (24).

The DNA sequence of *hrpW* was also determined for *P. syringae* pv. syringae B728a, revealing a 1,326-bp ORF. The gene is also preceded by a consensus *hrp* promoter, followed by a rho-independent transcription terminator, and appears to be in a monocistronic operon. The predicted protein of the B728a *hrpW* is also glycine rich, lacks cysteine, and has a harpin domain (amino acids 1 to 204) and a Pel domain (amino acids 205 to 441). The Pel domains of the two HrpW proteins are 85% identical, and all eight amino acids changed from the conserved cysteine are identical between the two HrpW proteins. In contrast, the harpin domains are only 53% identical. The region consisting of amino acids 121 to 204 contains seven imperfect glycine-rich repeats, one more than the DC3000 HrpW. The B728a repeats contain amino acids similar to those of the DC3000 HrpW repeats, but they do not have the TPS/DAT motif and contain an aspartic acid residue in the glycine-rich region of the repeat (Fig. 1C). Even though the repeat sequence is different, the predicted structure is the same. The accompanying paper by Kim and Beer (31) reports the cloning of a homologous gene, also designated *hrpW*, from *E. amylo-*

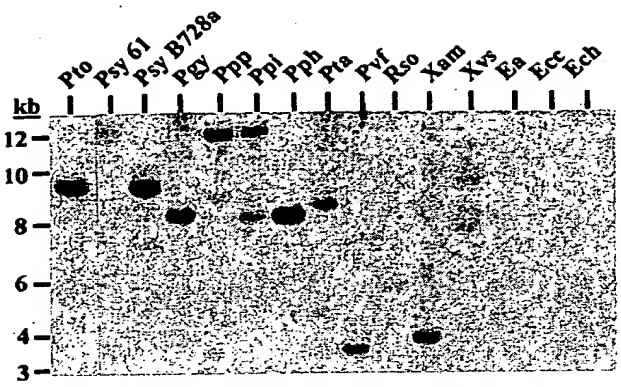

FIG. 2. DNA gel blot analysis of the hybridization of *hrpW* under high-stringency conditions to total DNA from other bacterial plant pathogens. DNA from the indicated pathogens was isolated, digested with *Eco*RI, resolved on a 0.5% agarose gel, transferred to an Immobilon-N membrane, and hybridized with a ^{32}P-labeled *hrpW* subclone at 62°C. Abbreviations: Pto, *P. syringae* pv. tomato; Psy, *P. syringae* pv. syringae; Pgy, *P. syringae* pv. glycinea; Ppp, *P. syringae* pv. papulans; Ppi, *P. syringae* pv. pisi; Pph, *P. syringae* pv. phaseolicola; Pta, *P. syringae* pv. tabaci; Pvf, *P. viridiflava*; Rso, *Ralstonia solanacearum*; Xam, *Xanthomonas campestris* pv. amoraciae; Xvs, *X. campestris* pv. vesicatoria; Ea, *Erwinia amylovora*; Ecc, *E. carotovora* subsp. *carotovora*; Ech, *E. chrysanthemi*.

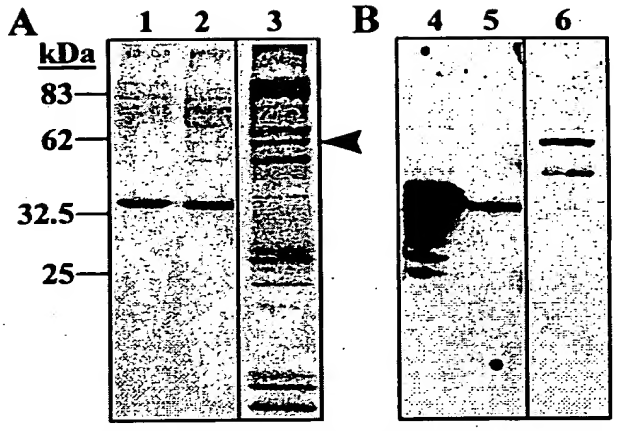

FIG. 3. SDS-PAGE and immunoblot analysis of preparations containing the *P. syringae* pv. tomato HrpW and its harpin domain and Pel domain fragments. (A) His$_6$-tagged full-length HrpW and the two domain fragments were partially purified by Ni-NTA chromatography, separated by SDS-PAGE, and stained with Coomassie blue R250. The arrowhead indicates the full-length HrpW, which is produced in very small amounts. Lanes: 1, Pel domain fragment; 2, harpin domain fragment; 3, HrpW. (B) The same HrpW derivatives were also visualized on immunoblots with anti-HrpW antibodies used in conjunction with the Western light chemiluminescence assay. Lanes: 4, Pel domain fragment; 5, harpin domain fragment; 6, HrpW.

vora. The DC3000 and *E. amylovora* HrpW proteins are 42.9% identical in their Pel domains but only 27.7% identical in their harpin domains.

***hrpW* appears widely distributed in plant-pathogenic bacteria and is in a region conserved between two *P. syringae* pathovars.** We further examined the distribution of *hrpW* by DNA gel blot analysis. The *P. syringae* pv. tomato DC3000 *hrpW* ORF was amplified by PCR and used as a probe for high-stringency gel blot hybridization with *Eco*RI-digested DNA from representative necrogenic gram-negative plant pathogens. The *hrpW* probe hybridized to at least one distinct band for each of the *P. syringae* pathovars tested: *glycinea* race 4 U1, *papulans* Psp19, *pisi* H27, *phaseolicola* 343, *tabaci* ATCC 11528, and *syringae* strains B728a and 61 (weakly). Hybridization was also observed with *Pseudomonas viridiflava* PV5, *Ralstonia* (*Pseudomonas*) *solanacearum* CR10 (weakly), and *Xanthomonas campestris* pathovars *amoraciae* XC-4 and *vesicatoria* H44 (Fig. 2). No hybridization was observed with DNA from *E. amylovora* Ea321, *E. carotovora* subsp. *carotovora* Ecc71, or *E. chrysanthemi* CUCPB5047.

HrpW and its harpin domain elicit an HR-like necrosis in tobacco leaves. PCR subclones of the *P. syringae* pv. tomato DC3000 *hrpW* were constructed in pQE30 to permit production of derivatives of HrpW and the two domain fragments carrying N-terminal His$_6$ tags. These fusion proteins were partially purified by Ni-NTA chromatography and analyzed by SDS-PAGE and immunoblotting with antibodies raised against *P. syringae* pv. tomato DC3000 Hrp-secreted proteins (Fig. 3). Anti-HrpW antibodies bound to the full-length HrpW and to both fragments, but binding to the harpin domain fragment was noticeably weaker. Transformants producing HrpW were highly unstable in their maintenance of the plasmid. Thus, HrpW levels were quite low, and Ni-NTA chromatography yielded a preparation that was only partially enriched in HrpW. Nevertheless, the HrpW preparation elicited an HR-like necrosis in tobacco leaves, which visibly differed from the necrosis elicited by the *P. syringae* pv. syringae 61 HrpZ only in developing ca. 12 h later (Fig. 4). The elicitor activity was heat stable and protease sensitive, and vector control preparations produced no response (data not shown). The partially purified harpin domain fragment also elicited a necrosis that was slightly delayed, and this response, like that elicited by HrpZ, could be inhibited by 1.0 mM lanthanum chloride, a calcium channel blocker (Fig. 4). Thus, the necrosis elicited by the HrpW harpin domain is an active plant response. In contrast, purified *E. chrysanthemi* PelE, obtained from *E. coli* JA-221 (pPEL748) (29), elicited a black, macerated necrosis that was not inhibited by 1.0 mM lanthanum chloride, 50 μM sodium vanadate, or 100 μM cycloheximide (data not shown). This is consistent with the expectation that pectic enzymes kill by lysis of turgid protoplasts through weakened cell walls rather than by elicitation of cell death programs. The HrpW Pel domain elicited no visible response in the infiltrated tobacco tissue.

The HrpW Pel domain binds calcium pectate but lacks detectable Pel activity. HrpW, its harpin domain, and its Pel domain were tested for Pel activity with the A_{230} assay for 4,5-unsaturated pectic products (13). No activity was detected despite trying both polygalacturonic acid and a 31% methylesterified derivative as substrates, $CaCl_2$ and $MnCl_2$ as cofactors, and several pH levels. Although the HrpW Pel domain lacks detectable Pel activity, it did bind to calcium pectate beads, even in the presence of 500 mM NaCl, at both pH 5.6, the pH of the plant apoplast, and pH 8.0, the optimal pH for activity of active Pels (Fig. 5). Full-length HrpW was not tested in these binding experiments because of problems in producing the protein in *E. coli*, as described above. The HrpW Pel domain was solubilized from beads by addition of EDTA or soluble pectate, both of which dissolve the beads. The protein did not precipitate when mixed with either $CaCl_2$ or sodium pectate individually. The HrpW Pel domain did not bind to calcium alginate beads prepared in the same manner as the pectate beads, indicating the binding is specific to pectate. Boiling the HrpW Pel domain for 10 min did not decrease its subsequent ability to bind pectate; thus the pectate binding and the HR eliciting activities of HrpW are both heat stable. HrpZ and marker proteins bracketing the pI and molecular weight of

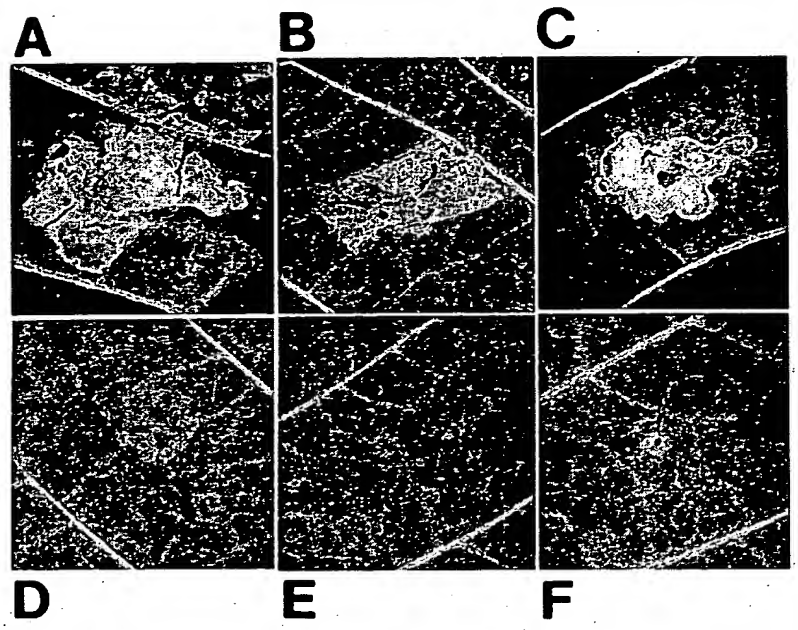

FIG. 4. Elicitation in tobacco leaves of active tissue death indicative of the HR by cell-free preparations containing the *P. syringae* pv. tomato HrpW and its N-terminal fragment. The protein preparations analyzed in Fig. 3 were infiltrated into tobacco leaves, in some cases with 1.0 mM lanthanum chloride. Leaves were photographed 48 h later. (A) *P. syringae* pv. syringae 61 HrpZ (0.12 μg/ml); (B) HrpW; (C) harpin domain fragment of HrpW (0.22 μg/ml); (D) HrpZ plus lanthanum chloride; (E) HrpW plus lanthanum chloride; (F) Pel domain fragment of HrpW (1.40 μg/ml).

the HrpW Pel domain were tested for calcium pectate binding, and none bound under these conditions (Fig. 5).

The ability of a *P. syringae* pv. tomato DC3000 *hrpZ hrpW* mutant to elicit the HR is substantially reduced. Marker exchange mutagenesis was used to construct *P. syringae* pv. tomato mutants CUCPB5094 (Δ*hrpZ*::*nptII*), CUCPB5096 (*hrpW*::ΩSp^r), and CUCPB5095 (Δ*hrpZ*::*nptII hrpW*::ΩSp^r). The Δ*hrpZ*::*nptII* mutation is nonpolar. All mutant constructions were confirmed by DNA gel blotting and immunoblotting (data not shown). Tobacco leaves were infiltrated with *P. syringae* pv. tomato DC3000 and the three mutant derivatives at two levels of inoculum and then examined 48 h later to determine the percentage of infiltrated tissue that was necrotic (Table 1). Only the *hrpZ hrpW* mutant was significantly reduced in the frequency with which it elicited collapse of more than 50% of the infiltrated area. We were unable to complement this phenotype since expression of *hrpW* or *hrpZ* in *trans* reduces the HR elicited by wild-type *P. syringae* pv. tomato DC3000. To determine if this mutant was reduced in virulence, tomato leaves inoculated with the mutant and wild-type DC3000 were monitored for symptom production and bacterial multiplication over a period of 5 days. No difference was observed (data not shown).

DISCUSSION

To identify an anticipated second harpin in *P. syringae*, we screened DNA in the *P. syringae* pv. tomato DC3000 *hrp* gene region for genes with harpin-like phenotypes. *hrpW* had the expected but paradoxical phenotype of interfering with HR elicitation when expressed in *trans*. This interference was not due to repression of the *hrpZ* operon, since HrpZ was expressed. It is more likely related to the blocking of HrpZ secretion, which we subsequently observed. HrpW was found to be identical to the previously identified and partially sequenced transcriptional unit V and to encode the previously identified Hrp-secreted protein EXP-60 (38, 49). In a preliminary report, this protein was referred to as HopPtoA (11). It is now designated HrpW based on its HR elicitor activity, the phenotype of a *hrpZ hrpW* mutant, and the homology of the protein with the HrpW proteins from *E. amylovora* strains Ea321 (31) and CFBP1430 (18). The latter report appeared when this article was under revision.

HrpW has several general characteristics of harpins, including amino-acid composition, heat stability, unexpectedly low mobility in SDS-PAGE, and the ability of both full-length and truncated proteins to elicit the HR (1, 2, 23, 47). HrpW also has six glycine-rich repeats that are similar to a repeated sequence found in HrpZ and are reminiscent of the repeat-rich structure of HrpZ (1). The general lack of cysteine residues in harpins is particularly striking in HrpW because comparison with the homologous fungal and bacterial Pels reveals that all eight of the conserved cysteine residues in those proteins have been substituted in HrpW. All of these properties raise the possibility that harpins, like the *Salmonella typhimurium* FlgM protein, may be in an unfolded state in the absence of their substrates or targets (16). This appears to be important to FlgM because of spatial constraints on the movement of globular proteins through the flagellum. With harpins, an unfolded state is more likely important for penetration into the plant cell wall matrix than for translocation through the Hrp pathway, since several Avr proteins thought to travel the pathway are relatively large and cysteine rich.

The ability of isolated *P. syringae* HrpZ and HrpW proteins to elicit the HR when infiltrated into tobacco leaf tissue may not directly reflect biological function because the Avr proteins now appear to be both essential and sufficient (once delivered to the plant cytoplasm) for elicitation of the bacterial HR (2).

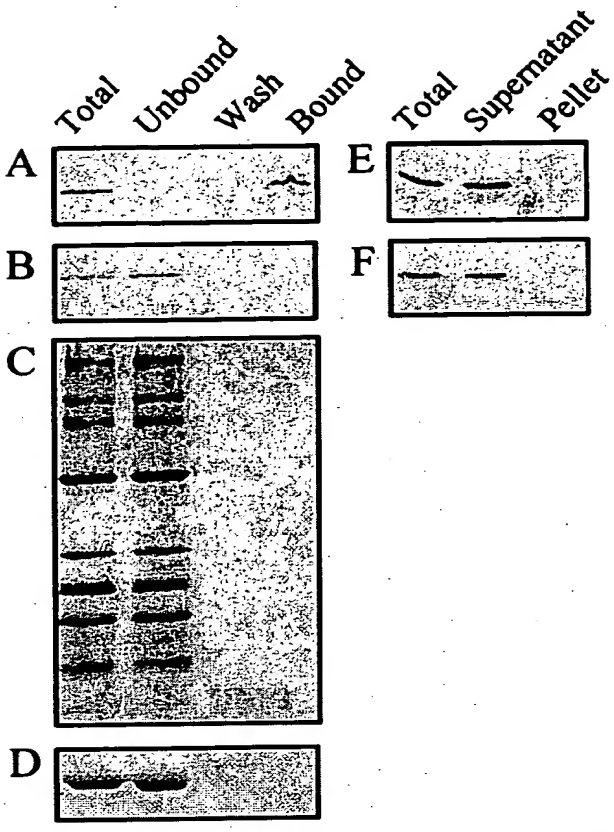

FIG. 5. Binding of the HrpW Pel domain to calcium pectate beads. Purified HrpW Pel domain or marker proteins were mixed with calcium pectate or calcium alginate beads in 50 mM MES (pH 5.6), 500 mM NaCl and washed several times with the same buffer. In panels A through D, equal volumes from the original protein-bead mix (Total), the supernatant from the original mix (Unbound), the last buffer wash (Wash), and the washed beads (Bound) were analyzed by SDS-PAGE to determine if proteins bound to calcium pectate or calcium alginate beads. (A) HrpW Pel domain with calcium pectate beads; (B) HrpW Pel domain with calcium alginate beads; (C) mixed low-molecular-weight, low-pI, and high-pI marker proteins (Pharmacia, Uppsala, Sweden) with calcium pectate beads; (D) HrpZ with calcium pectate beads. In panels E and F, equal volumes from the original protein mix (Total), the supernatant, and the pellet were similarly analyzed to determine protein precipitation. (E) HrpW Pel domain with $CaCl_2$; (F) HrpW Pel domain with soluble pectate.

Our data do not permit us to compare the relative efficacy of HrpZ and HrpW to elicit the HR, but it is worth noting that harpins remain the only bacterial proteins known to elicit apparent programmed plant cell death when exogenously applied.

Harpins appear to have a site of action in plant cell walls. HrpZ associates with the walls rather than the membranes of plant cells, and the protein elicits no response from wall-less protoplasts (25). The Pel domain in HrpW binds to pectate (in the presence of calcium), which is the component controlling porosity of the cell wall matrix (6). This binding occurs at a physiological pH that is above the pK of polygalacturonic acid (pectate) and the predicted pI (4.9) of the HrpW Pel domain, which eliminates binding attributable to a net charge difference between the two molecules. The failure of polymannuronic acid (alginate) to bind the HrpW Pel domain provides further evidence for the specificity of pectate binding. The Pel domain does not have elicitor activity, and its presence does not prevent native HrpW from having this activity. This suggests that HrpW elicitor activity resides in an interaction with the plant cell wall, or at least that it can occur despite immobilization in the pectic matrix of the wall. Membership of the homologous *E. amylovora* HrpW in a newly defined class of fungal and bacterial Pels is discussed in the accompanying paper by Kim and Beer (31). Neither of these HrpW proteins has detectable Pel activity. Pel homologs with no detectable activity in vitro are also found in the pollen and style tissues of several plants, and the conservation of catalytic residues in these proteins suggests a cryptic enzymatic function (24).

Mutation of transcription unit V reduced neither the HR nor the virulence phenotypes of *P. syringae* pv. tomato DC3000 (38), and our *hrpZ hrpW* mutant was significantly reduced only in its HR phenotype (although our virulence assay would likely miss a subtle reduction). Because the expression of *hrpZ* and *hrpW* in *trans* interferes with HR elicitation, even in wild-type cells, we were unable to perform standard complementation tests with our *hrpZ hrpW* double mutant. However, the construction of each mutation was confirmed by sequence analysis, and the full virulence of the double mutant further argues against second-site mutations. One interpretation of the observation that *hrpZ* mutation strongly reduces HR elicitation by *E. coli*(pHIR11) and *P. fluorescens*(pHIR11) (1, 19), whereas *hrpZ hrpW* double mutation only weakly reduces HR elicitation by *P. syringae* pv. tomato, is that *P. syringae* produces additional harpin-like proteins, analogous to the multiple Pel isozymes of *E. chrysanthemi* and *E. carotovora* (7).

The presence of sequences hybridizing with *hrpW* in several other plant-pathogenic bacteria, particularly *P. viridiflava* and *X. campestris*, is significant because it suggests that HrpW has some broadly important function, and it implies that *P. viridiflava* has a Hrp system and that *X. campestris* produces a harpin. Although the Hrp system of *X. campestris* has been extensively characterized, no harpin or any other protein has been found to be secreted by the Hrp system in culture. *hrpW* should be useful as a probe to clone a gene encoding such a protein from *X. campestris*. The function of harpins and their possible involvement in the delivery of Avr proteins through plant cell walls remains a matter of speculation. However, our finding of a *P. syringae* harpin with a Pel domain and calcium pectate binding activity provides further evidence that Avr proteins and harpins differ in their sites of action, with many Avr proteins acting inside plant cells and harpins acting outside, probably in the cell wall.

TABLE 1. Reduced frequency of HR elicitation in tobacco leaves by *P. syringae* pv. tomato DC3000 *hrpZ* and *hrpW* mutants

P. syringae pv. tomato strain	Relevant genotype	Frequency of HR[a]	
		Inoculum of 1×10^7 cells/ml	Inoculum of 5×10^7 cells/ml
DC3000	Wild type	17/19	18/18
CUCPB5094	Δ*hrpZ*::*nptII*	12/15	13/13
CUCPB5096	*hprW*::ΩSp^r	13/15	13/13
CUCPB5095	Δ*hrpZ*::*nptII hrpW*::ΩSp^r	7/19[b]	15/18

[a] The number of inoculated panels showing more than 50% collapse 48 h after inoculation relative to the total number inoculated.

[b] This value differs significantly (binomial test) from that of the wild type ($P = 0.01$). No others differ significantly from the wild type ($P = 0.05$).

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